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A Novel Family of Putative Pheromone Receptors in Mammals with a Topographically Organized and Sexually Dimorphic Distribution

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Summary

Mammals have retained two functionally and anatomically independent collections of olfactory neurons located in the main olfactory epithelium and in the vomeronasal organ (VNO). Pheromones activate the VNO in order to elicit fixed action behaviors and neuroendocrine changes involved in animal reproduction and aggression. Differential screening of cDNA libraries constructed from individual rat VNO neurons has led to the isolation of a novel family of ~100 genes encoding seven transmembrane receptors with sequence similarity with Ca^{2+}-sensing and metabotropic glutamate receptors. These genes are likely to encode a novel family of pheromone receptors. Patterns of receptor gene expression suggest that the VNO is organized into discrete and sexually dimorphic functional units that may permit segregation of pheromone signals leading to specific arrays of behaviors and neuroendocrine responses.

Introduction

Animals have evolved chemosensory systems that are able to discriminate a large array of natural scents and to translate olfactory stimuli into complex sensory information: the presence of food, the anticipation of a danger, the approach of a mate. In terrestrial vertebrates, independent collections of chemosensory neurons and neuronal networks process two basic modalities of smell: sensory discrimination of odorant molecules and pheromone perception. The main olfactory system is able to recognize and discriminate the large array of molecules commonly defined as odorants. This olfactory information is transmitted from the main olfactory epithelium (MOE) lining the posterior recess of the nasal cavity (Figure 1) to the main olfactory bulb (MOB) and to cortical and neocortical centers of the brain, where olfactory signals are processed and integrated, generating cognitive and behavioral responses such as aversive or pleasurable responses to a smell.

The second set of chemosensory neurons resides in the vomeronasal organ (VNO), a tubular structure of the nose enclosed within bilateral bony capsules of the ventral septum (Jacobson, 1811). Axonal projections from the VNO fasciculate to form the vomeronasal nerve and reach target cells within the accessory olfactory bulb (AOB) (Figure 1). Neuronal tracing experiments have shown that the VNO is exclusively connected to specialized centers of the limbic system, including the vomeronasal amygdala, the bed nucleus of the stria terminalis, and specific nuclei of the ventromedial hypothalamus involved in reproduction and aggression (Halpern, 1987, and references therein). Thus, in contrast to odor discrimination by the MOE, pheromone perception by the vomeronasal system results in behavioral and endocrine responses that do not involve higher cognitive centers of the brain.

Surgical ablation of the VNO severely impairs recognition of chemical cues broadly defined as pheromones, which are present in urine, sweat, and other bodily secretions and are involved in chemical communication among animals (reviewed by Halpern, 1987; Wysocki, 1989). Pheromones in mammals convey specific information concerning the species, gender, and identity of the animal in order to trigger stereotyped behavioral and neuroendocrine responses. These responses ensure breeding and hierarchical order in the animal group and are primarily mediated by the VNO with a contribution from the main olfactory system. In most instances, the exact nature of mammalian pheromones has not yet been elucidated.

Recent molecular advances have demonstrated that MOE and VNO sensory neurons utilize unrelated sets of genes to translate the olfactory information into electrical stimuli (Dulac and Axel, 1995; Liman, 1996), suggesting that, despite a common location in the nose and a common embryonic origin in the olfactory placode, the two chemosensory systems might have evolved from independent ancestral sensory systems. In mammals, odorant receptors are G protein–coupled seven transmembrane domain receptors encoded by a family of several thousand genes (Buck and Axel, 1991). The olfactory epithelium contains several million bipolar olfactory sensory neurons, each projecting to one of several thousands of glomeruli in the main olfactory bulb and establishing synapses with dendrites of mitral and tufted cells (Figure 1). Mitral and tufted cells in turn send axons to the olfactory cortex, which further dispatches the olfactory information to higher cognitive centers. Convergent lines of evidence strongly suggest that individual MOE sensory neurons express only one of the thousand receptor genes (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; C. D. and R. Axel, unpublished data). Moreover, all neurons expressing the same receptor and therefore responsive to a small subset of odors, although randomly distributed in domains of the epithelium, project their axons to one or a small number of discrete loci or glomeruli within the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). The positions of specific glomeruli are topographically fixed and are conserved in the brains of all animals within a species. These data provide physical evidence that the olfactory bulb defines a two-dimensional map that identifies which of the numerous receptors have been activated within the sensory epithelium. Thus, the quality of an olfactory stimulus is likely to be encoded by the specific combination of glomeruli activated by a given odorant.

The isolation of the genes encoding the pheromone receptors from the VNO might similarly provide significant insight into the specific organization of neurons

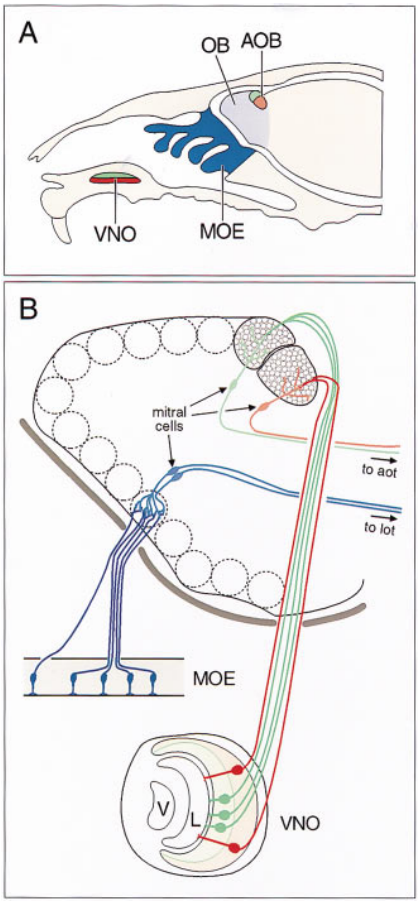

Figure 1. Anatomical Organization of the VNO and the Main Olfactory System

(A) Drawing of a parasagittal section of an adult rat head. Neurons from the main olfactory epithelium (MOE) project to the olfactory bulb (OB). VNO neurons are segregated into two neuronal populations distinguished by differential expression of the G-protein subunits $G\alpha_{i2}$ (indicated by green color) and G_o (indicated by red color) and are likely to project to different areas of the accessory olfactory bulb (AOB).

(B) Schematic representation of VNO and MOE axonal projections. In the bulb, MOE and VNO axons form neuropils called glomeruli where they synapse with mitral cells. OB and AOB mitral cells project to distinct centers of the brain. aot, accessory olfactory tract; lot, lateral olfactory tract.

in the vomeronasal system that translate pheromone signals into stereotyped and sexually dimorphic behavioral responses.

We recently developed a cloning strategy that led to the isolation of genes likely to encode mammalian pheromone receptors (Dulac and Axel, 1995). Differential screening of cDNA libraries constructed from single sensory neurons from the rat VNO has enabled us to isolate a family of genes encoding a novel family of seven transmembrane domain receptors unrelated to the receptors expressed in the MOE and to other known seven transmembrane domain receptors. However, transcripts isolated by virtue of sequence homology with the family of VNO receptor genes were consistently expressed in a restricted area of the vomeronasal neuroepithelium closest to the apical surface. Subsequent efforts to identify the full repertoire of vomeronasal receptor genes that are presumably expressed in other regions of the vomeronasal epithelium have been unsuccessful, supporting the idea that the receptors we had initially isolated accommodated only a subset of VNO neurons. Independent studies have shown that distribution of several markers, including potential components of signal transduction pathways, is regionalized in nonoverlapping populations of vomeronasal sensory neurons (Shinohara et al., 1992; Halpern et al., 1995; Berghard and Buck, 1996). For example, the G proteins $G\alpha_{i2}$ and G_o are expressed in the apical and basal halves of the VNO neuroepithelium, respectively, and immunocytochemistry of the accessory bulb indicates that fibers reaching the accessory bulb are likely to stay segregated according to their origin from the apical or basal sides of the VNO (Figure 1). This together with our data suggests that the VNO maintains a dual structure for the processing of pheromone signals and incited us to search for a second family of pheromone receptors in the VNO neuroepithelium.

Differential screening of a cDNA library prepared from individual VNO neurons led us indeed to the identification of a novel family of ~100 genes that present a topographically organized and sexually dimorphic pattern of expression. These newly discovered genes encode putative receptors that show sequence similarities with a superfamily of G-coupled seven transmembrane domain receptors, which include the Ca^{2+}-sensing receptor and metabotropic glutamate receptors. Thus, this novel class of VNO receptors has a molecular structure that is evolutionarily independent of the odorant receptors of the MOE and of the first identified family of putative pheromone receptors and hence is likely to represent a second and independent family of mammalian pheromone receptors.

Results

The VNO Neuroepithelium Expresses Two Independent Families of Pheromone Receptors

We hypothesized the existence of two distinct families of genes encoding pheromone receptor genes that are selectively colocalized with either the $G\alpha_o$ protein in the basal half of the vomeronasal neuroepithelium or with the $G\alpha_{i2}$ protein in the apical region. For simplicity of nomenclature, and with the understanding that the cosegregation of distinct G-protein α subunits with independent families of pheromone receptors is consistent but does not demonstrate a functional link, the family of genes encoding putative pheromone receptors that we have previously identified and that colocalize with $G\alpha_{i2}$ will be named $G\alpha_{i2}$-VN, whereas the novel family of receptors coexpressed with G_o and described in this study will be named G_o-VN.

In the absence of information concerning the nature of the G_o-VN receptor molecules, we reiterated the cloning strategy that allowed us to identify a family of putative pheromone receptor genes expressed by $G\alpha_{i2}^+$ neurons (Dulac and Axel, 1995). This strategy was based on the

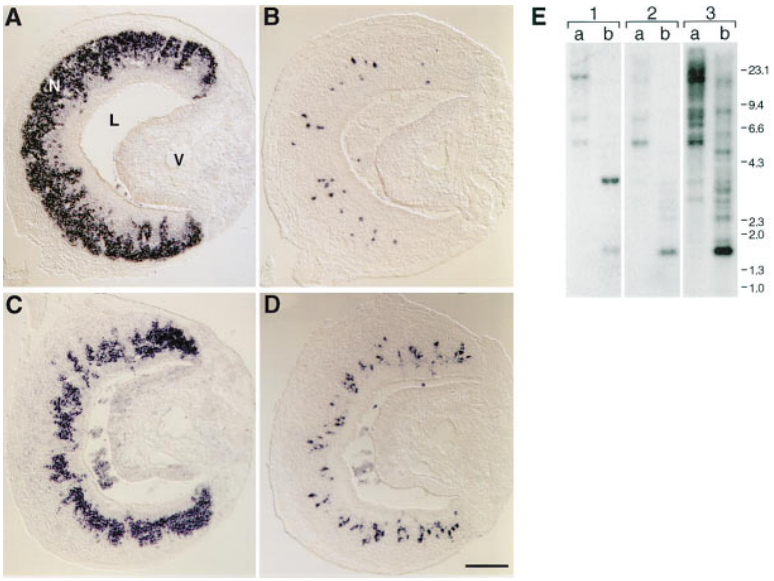

Figure 2. Identification of a Novel Receptor Gene Family Specifically Expressed by VNO Neurons Coexpressing the G Protein G_o

(A–D) Coronal sections of VNOs dissected from adult male rats were hybridized with antisense RNA probes for G protein G_o (A), G protein $G\alpha_{i2}$ (C), a mix of pheromone receptor probes ($G\alpha_{i2}$-VN) expressed by $G\alpha_{i2}^+$ neurons (D), and the cDNA G_o-VN13b identified by differential screening of a cDNA library prepared from an isolated VNO neuron (B).

(E) Rat genomic DNA was digested with EcoR1 (a) and BamH1 (b) and analyzed on Southern blots after hybridization with G_o-VN13a (1) and G_o-VN13b (2 and 3) probes under conditions of high stringency (1 and 2) and low stringency (3).

assumption that individual neurons within the VNO are likely to express only one pheromone receptor gene and that transcripts encoding a given receptor represent between 1% and 0.1% of a single-cell mRNA. Differential screening of cDNA libraries constructed from single-VNO neurons takes advantage of the fact that different cells express different receptors and thus provides an experimental solution to the problem of detecting a specific transcript in a heterogeneous population of neurons. In this attempt, we expected that differential screening of a cDNA library prepared from an isolated G_o^+, $G\alpha_{i2}^-$ VNO neuron would permit the isolation of a class of pheromone receptor genes distinct from the $G\alpha_{i2}$-VN family of receptor genes.

A cDNA library prepared from a G_o^+ neuron (VN13) was differentially hybridized with ^{32}P-labeled probes prepared from VN13 and from a second VNO neuron cDNA (VN10). A 425 bp cDNA (G_o-VN13A) present at a frequency of 0.1% in the VN13-cDNA library showed selective hybridization with VN13 cell probe. Two cDNAs of longer size, G_o-VN13B and G_o-VN13C, were subsequently isolated from a cDNA library prepared from dissected adult VNOs and showed 90% sequence similarity with G_o-VN13A. Hybridization to VNO cross-sections with digoxigenin-labeled antisense RNA probe showed that expression of these transcripts is restricted to a small subpopulation of VNO neurons in a location consistent with the region of G_o expression of the neuroepithelium (Figure 2). The sequence of G_o-VN13B reveals a partial open reading frame that includes seven hydrophobic stretches of ~20 amino acids in length. G_o-VN13B sequence does not share any resemblance with the odorant receptor genes nor with the family of putative pheromone receptor genes previously identified (see below). In addition, hybridization of G_o-VN13B DNA probe to genomic DNA identified two discrete bands at high stringency and 13 or more at lower stringency (Figure 2E), revealing the existence of a family of closely related genes in the rat genome.

Taken together, these data indicate that we have isolated a novel multigene family encoding seven transmembrane domain receptors and expressed by subsets of VNO neurons from the basal half of the neuroepithelium.

Sequence of a New Family of VNO Receptors

Recombinant phages from a VNO cDNA library were screened at low stringency with the G_o-VN13B DNA probe. Six distinct gene subfamilies were isolated that showed no cross-hybridization under stringent conditions of hybridization and washing. cDNAs G_o-VN1 to G_o-VN6, each representative of a subfamily, were fully sequenced (Figure 3). In G_o-VN1 to G_o-VN5 cDNAs, the first methionine of the open reading frame was tentatively chosen as a start for protein translation, revealing large open reading frames ranging from 548 to 866 amino acids. A frame shift in the G_o-VN6 sequence (amino acid 532; indicated by slash bar in Figure 3) indicated that this transcript is unable to generate a functional protein.

Hydropathy analysis of the predicted G_o-VN proteins with the Kyte-Doolittle algorithm identified a large hydrophilic N-terminal domain that ranges in size from 274 amino acids in G_o-VN1 to 595 in G_o-VN4. This is preceded in cDNAs G_o-VN4, G_o-VN7, and G_o-VN13C by an initial hydrophobic 21 amino acid segment characteristic of eukaryotic signal sequences (von Heijne, 1986). A cluster of seven hydrophobic regions representing potential membrane-spanning helices and typical of the G protein–coupled receptor superfamily is followed by a short hydrophilic sequence that indicates a potential intracytoplasmic C-terminal domain.

A database search indicated the presence of sequence motifs common to Ca^{2+}-sensing and metabotropic glutamate (mGluR) receptors (Houamed et al., 1991; Masu et al., 1991; Brown et al., 1993; Pollak et al., 1993). Pairwise sequence alignments reveal 18% to 23% sequence identity between the rat Ca^{2+}-sensing receptor and the most distant (G_o-VN3) and the closest (G_o-VN1) G_o-VN sequences, respectively. Sequences of rat mGluR1 and G_o-VN cDNAs appear more distantly related. Several localized regions showed a more pronounced degree of similarity, including a cysteine-rich

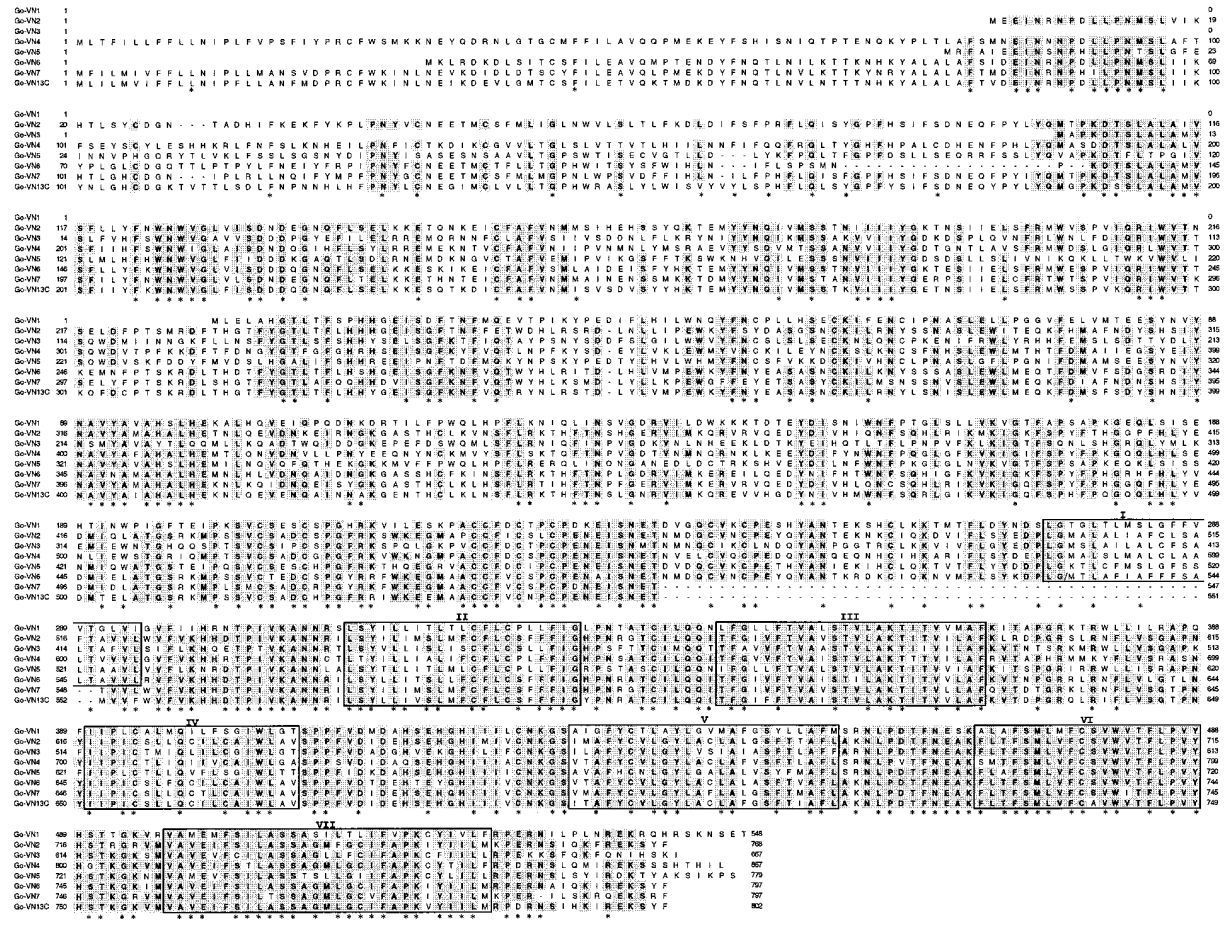

Figure 3. Amino Acid Sequences of cDNAs from the G_o-VN Family of Pheromone Receptors

The deduced amino acid sequences of eight cDNAs belonging to the G_o-VN family of putative pheromone receptors. Predicted position of seven transmembrane domains is indicated (I–VII). Amino acids common to at least five cDNAs are shaded. Amino acids common to the rat mGluR1 and Ca^{2+}-sensing receptors are indicated by a star.

sequence just preceding the first transmembrane domain (amino acid 206 to 260 in G_o-VN1), the predicted transmembrane domains 2 to 7 with surrounding cytoplasmic and extracellular loops, and the relative position of 20 cysteines. The N-terminal and first transmembrane domains show little degree of homology. In mGluR and Ca^{2+}-sensing receptors, the second intracellular loop is involved in providing specificity for G-protein coupling (Gomeza et al., 1996), enabling different classes of mGluR receptors to activate phospholipase C or to inhibit adenylyl cyclase. In G_o-VN, this domain is rich in basic residues, as expected for potential G-protein coupling, and shows closer resemblance to the class II and III mGluRs that were shown to couple to G_o and G_i α subunits.

Overall, the six G_o-VN sequences share between 42% and 75% sequence identity. Regions of G_o-VN proteins downstream of transmembrane domain 2 are nearly identical in all VNO receptor sequences. In contrast, N-terminal extracellular regions and first transmembrane domains are quite divergent.

Anomalies in G_o-VN cDNA Sequences

Two unusual features were observed in the sequence of some G_o-VN cDNAs. In G_o-VN1 and G_o-VN3 cDNAs, stretches of open reading frame can be found in the 5′ extremity of the cDNAs that generate polypeptide sequences of 310 and and 152 amino acids, respectively, which are interrupted by a frameshift in G_o-VN1 and by an insertion of 500 nucleic acids in G_o-VN3. The prospective receptor protein sequences indicated for G_o-VN1 and G_o-VN3 (Figure 3) start at the next available methionin and are therefore significantly shorter than those of other receptor cDNAs. Further studies will be required to assess whether protein translation can indeed be initiated at the position indicated and whether receptor proteins hence generated are functional.

G_o-VN7 and G_o-VN13c cDNAs show a similar deletion of 150 bp located at the exact same position in the sequence. Strikingly, the 150 bp deletion does not alter the open reading frame but generates a gap that encompasses 34 amino acids upstream of the first transmembrane domain and most of the first transmembrane domain itself. Hydropathy analysis of G_o-VN7 and G_o-VN13c protein sequences detects only a seven to eight amino acid long hydrophobic stretch that might not be long enough to replace the deleted transmembrane domain 1 and allow the appropriate folding of the protein. Except for the 150 bp gap, sequences of G_o-VN13B and G_o-VN13C are identical. This raises the question as to

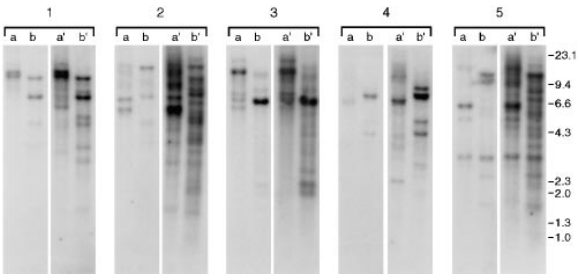

Figure 4. Southern Blot Analysis with Divergent Subfamilies of G_o-VN Receptors

Rat genomic DNA was digested with EcoR1 (a and a′) and BamH1 (b and b′) and analyzed by Southern blots after hybridization with ^{32}P-labeled probes corresponding to five divergent G_o-VN cDNAs (1, G_o-VN1; 2, G_o-VN2; 3–5, G_o-VN4 to G_o-VN6) under conditions of high (a and b) and low stringency (a′ and b′).

whether both transcripts might originate from alternative splicing of the same gene. Alternatively, they might be transcribed from independent genes that evolved from recent duplication and deletion events. Additional experiments will be required to determine whether these deleted transcripts are able to generate functional proteins or whether they simply correspond to pseudogenes.

Size of the G_o-VN Family of Genes

We investigated the size of the G_o-VN family of receptors by hybridizing ^{32}P-labeled cDNA probes prepared from regions spanning the most divergent N-terminal half of the receptor protein to rat genomic DNA. Individual probes identify two to four discrete bands under stringent conditions of hybridization and washing. Under conditions of reduced stringency, each of the individual probes now generates a unique pattern of 12 to 20 bands (Figure 4), providing a direct illustration of the existence of a very large family of related genes.

A direct estimate of the size of the G_o-VN receptor gene family was obtained by low stringency screening of a rat genomic library. PCR amplification on genomic DNA had indicated that receptor genes are devoid of introns in the region encompassing transmembrane domains 2 to 7 (data not shown), enabling us to deduce directly the number of genes present in the rat genome. A mix of ^{32}P-labeled DNA probes prepared from the six G_o-VN cDNA fragments identified 110 positive clones per haploid genome, indicating that the family of G_o-VN receptors may consist of $\sim$100 genes.

Expression Pattern of G_o-VN Receptors

The pattern of expression of the G_o-VN receptor genes was examined by in situ hybridization with digoxigenin-labeled RNA antisense probes. No signal was observed after hybridizing the mix of G_o-VN1 to G_o-VN6 receptor probes to sections of muscle, testis, brain, or whole head. The adult olfactory epithelium was also consistently negative, although rare positive cells (one to three cells per section) were observed in the olfactory neuroepithelium of E19 rat embryo (data not shown).

In contrast, strong signals were observed when antisense receptor RNA probes were hybridized to VNO neuroepithelium. In adults, each one of the G_o-VN probes detects small subsets of VNO sensory neurons (Figure 5). When hybridization and washing were performed at lower temperature, the number of faintly labeled neurons increased significantly (Figure 5), revealing cross-hybridization to more distant receptor genes.

Under high stringency conditions, cDNA clones G_o-VN1 to G_o-VN6 label 1.9%, 3.6%, 6.1%, 0.4%, 3.5%, and 1.3% of the VNO sensory neurons, respectively. Under the same experimental conditions, the mix of all six G_o-VN RNA probes labels 19% of the cells. This number is similar to the sum of labeled neurons detected with the six individual G_o-VN probes (17%), indicating that probes representing the six receptor subfamilies recognize distinct populations of VNO sensory neurons.

Spatial Distribution of G_o-VN Receptor Transcripts

Positive neurons identified with each of the G_o-VN probes were randomly distributed along the anteroposterior and dorso-ventral axis of the VNO neuroepithelium. Most RNA probes recognize cells that are preferentially localized in the most basal two-thirds of the neuroepithelium corresponding to the zone of G_o expression (Figure 5).

However, careful examination of adjacent cross-sections of vomeronasal neuroepithelium labeled with each of the G_o-VN probes reveals a well-organized spatial distribution of receptor expression. Different receptors appear preferentially localized in radial zones that define a series of hemiconcentric rings of distinct diameters. This pattern is observed along the entire length of the VNO and is conserved in all animals analyzed. The G_o-VN3 probe, for example, recognizes a subset of neurons that are confined to the most basal third of the VNO neuroepithelium (Figure 5). In contrast, the G_o-VN1, G_o-VN4, and G_o-VN5 RNA probes identify cells restricted to a hemiconcentric zone immediately apical to the area of G_o-VN3 expression, whereas G_o-VN2 (in the section shown in the upper right corner of Figure 5) identifies cells apposed to the apical layer of supporting cells. G_o-VN6 in turn is found only in sparse cells immediately apposed to the basal membrane. This is best seen in a statistical representation of G_o-VN receptor localization collected from VNO sections and multiple animals that shows a striking conservation of these patterns (Figure 6).

Thus, transcription of G_o-VN cDNAs appears restricted to one of three circumscribed areas of the VNO neuroepithelium in a manner quite reminiscent of the odorant receptor gene expression in four zones of the MOE (Ressler et al., 1993; Vassar et al., 1993). Although G_o-VN3 and G_o-VN6 transcripts show a clear segregation in the most basal region of the VNO neuroepithelium, the sequence anomalies found in both transcripts leave the functionality of this area of the neuroepithelium as an open question.

Sexual Dimorphism in Receptor Distribution and Age-Related Changes

To identify potential sexual dimorphism in G_o-VN receptor expression, we systematically hybridized each probe to sections originating from adult male and female rat VNOs. All receptors were equally distributed in males and females with the striking exception of G_o-VN2. In

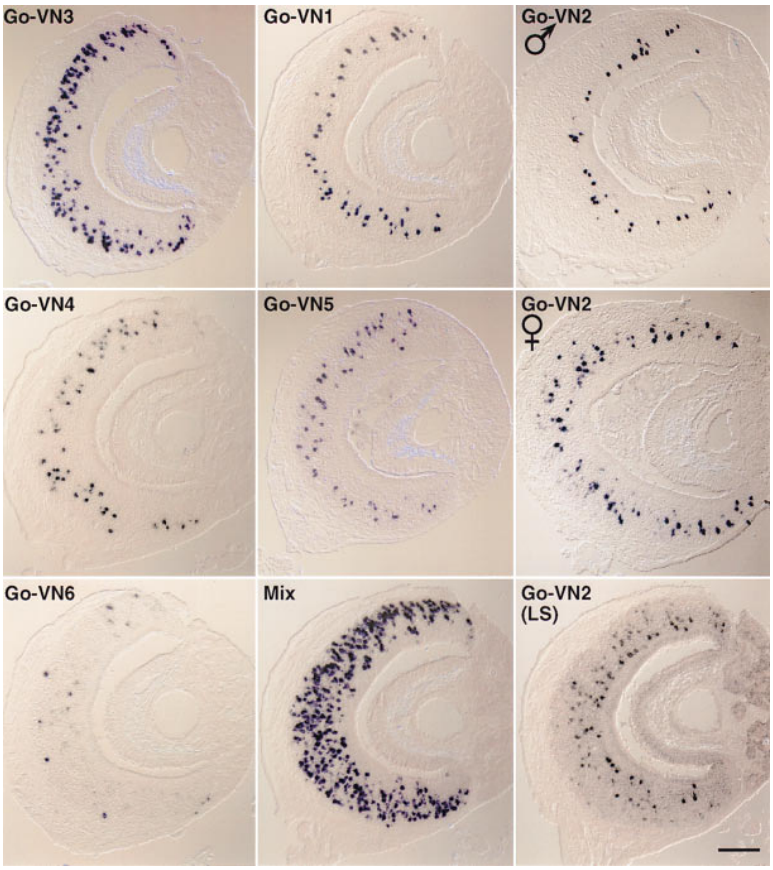

Figure 5. Expression and Localization of G_o-VN Receptor Transcripts in Coronal Sections of Adult Rat VNO

Cross-sections of VNO dissected from male or female adult rats were hybridized to digoxigenin-labeled antisense RNA probes under high and low (LS) conditions of stringency. Individual cDNA clones encoding for G_o-VN1 to G_o-VN6 and a mix of G_o-VN1 to G_o-VN6 cDNAs were used as templates. Scale bar = 120 μm.

females, G_o-VN2 appears expressed in a large and centrally located region comprising one-third of the neuroepithelium (Figures 5 and 6). In sharp contrast, the same probe recognizes in males a cohort of cells in the most apical side of the neuroepithelium, closely apposed to the VNO lumen, and most likely intermingled with $G\alpha_{i2}$ VNO sensory neurons (Figures 5 and 6). Such a difference in the G_o-VN2 expression pattern in males and females might result from the expression of the same receptor gene in a different zone of the VNO epithelium or from a differential expression of two distinct but closely related genes of the G_o-VN2 subfamily. In females, G_o-VN2 generates a very intense hybridization signal to most positive neurons and a fainter staining on a second set of labeled cells. The population of faintly labeled cells was never detected in males, indicating the existence of a female-specific neuronal subpopulation expressing either a lower level of the G_o-VN2 transcript or a female-specific receptor significantly different but still cross-hybridizing to the G_o-VN2 probe.

We followed the emergence of receptor expression and of the VNO zonal organization during development and postnatal stages preceding puberty. G_o-VN receptor expression is first detected in the VNO of E14 embryos. No significant difference is observed in the onset of expression of $G\alpha_{i2}$-VN and G_o-VN classes of receptor genes (data not shown). In agreement with data of Berghard and Buck (1996) in mouse, segregation of $G\alpha_{i2}$ and G_o expression in the apical and basal areas of VNO neuroepithelium, respectively, is not apparent in the embryo and in 1-week-old animals (Figure 7). In contrast, $G\alpha_{i2}^+$ cells appear randomly distributed in large clusters over the whole thickness of the neuroepithelium, intermingled with G_o cells. At 4 weeks after birth, however, $G\alpha_{i2}$ cells appear clearly localized in the apex of the epithelium. Similarly, in situ experiments with mixes of G_o-VN and $G\alpha_{i2}$-VN receptor probes on sections of the VNOs dissected from late embryos and 1-week-old animals (Figure 7) show that the two cell populations are still intermingled at early postnatal stages. We observed that the zonal distribution of the two families of receptors slowly emerges during sexual maturation to reach the spatial distribution observed in adults. Preliminary data indicate that the sexual dimorphic expression pattern of G_o-VN2 is undetectable at 6 weeks after birth (data not shown).

Thus, in contrast to the zones of olfactory receptor gene expression, which are already present in the olfactory epithelium at the earliest stages of receptor gene expression in the embryo (Sullivan et al., 1995), the spatial organization of the VNO neuroepithelium as detected by G-protein and receptor gene expression emerges only in a late postnatal period and reaches its definitive pattern at sexual maturity.

Expression of G_o-VN Receptors Is Restricted to G_o^+ VNO Neurons

The expression of some of the G_o-VN receptors in neurons lining the VNO lumen in an area mainly occupied by $G\alpha_{i2}^+$ cells raises the obvious question as to whether the expression of this family of genes is strictly restricted

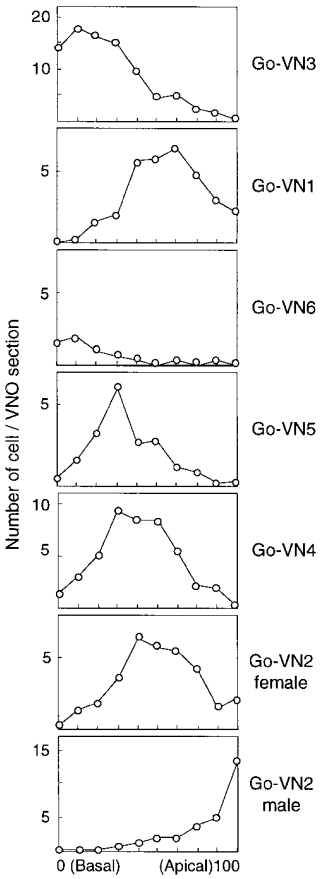

Figure 6. Analysis of the Spatial Distribution of G_o-VN1 to G_o-VN6 along the Basal–Apical Axis in Adult Rat VNOs

The basal–apical axis of each VNO section was divided into ten hemiconcentric rings of increasing diameters. Each point of the curve represents an average number of labeled cells per discrete zone after counting six to eight sections originating from four independent animals analyzed during two independent experiments. For G_o-VN2, 14 sections corresponding to ten individuals and four independent experiments were analyzed for each sex.

to G_o^+ VNO neurons. Single-cell cDNA prepared from 23 individual VNO neurons was analyzed by Southern blots with probes representing the six divergent subfamilies of G_o-VN receptors and was PCR amplified with degenerated primers based on conserved motifs between G_o-VN receptor sequences. Both approaches confirmed that none of the 19 cell cDNAs prepared from $G\alpha_{i2}^+$ neurons contained any sequence of the G_o-VN receptor family. In contrast, all four cDNAs generated from $G\alpha_{i2}^-$ cells contained a sequence related to the G_o-VN receptors. PCR products generated with degenerated primers based on conserved motifs between G_o-VN receptor sequences and obtained from the four G_o^+ cells were subcloned and sequenced. For each single-cell cDNA, the insert sequences from ten independent colonies were found to be identical.

This set of data strongly suggests that G_o-VN receptor genes are not expressed by $G\alpha_{i2}^+$ neurons and constitutes preliminary evidence for the expression of only one G_o-VN receptor gene per neuron.

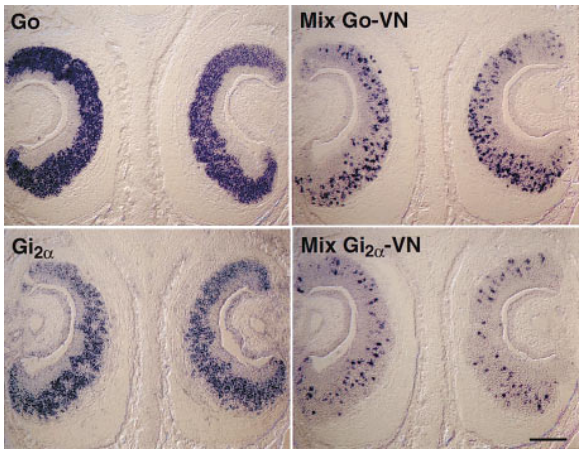

Figure 7. Spatial Distribution of VNO Sensory Neurons Expressing G_o, $G\alpha_{i2}$, G_o-VN, and $G\alpha_{i2}$-VN Receptors in Young Animals

In situ hybridization was performed on cross-sections through the head of 1-week-old rats. Antisense digoxigenin-labeled RNA probes were generated from cDNA encoding G_o, $G\alpha_{i2}$, and mix or G_o-VN and $G\alpha_{i2}$-VN receptors. Scale bar = 120 μm.

Discussion

We have isolated a novel family of ~100 genes encoding seven transmembrane domain receptors that shows topographically organized and sexually dimorphic patterns of expression in the vomeronasal neuroepithelium. These genes are likely to encode a novel family of mammalian pheromone receptors that may provide the molecular basis for translation of pheromone signals into specific arrays of sexual dimorphic behaviors and neuroendocrine responses.

Mature olfactory marker protein (OMP) positive VNO sensory neurons were shown to express one of the two G protein α subunits G_o and $G\alpha_{i2}$ according to their respective location in the basal or the apical half of the VNO neuroepithelium (Halpern et al., 1995; Berghard and Buck, 1996). Differential screening of single-cell cDNA libraries prepared from OMP^+, G_o^+ VNO neurons led us to discover a novel family of ~100 genes encoding putative seven transmembrane domain receptors (G_o-VN receptors) that are evolutionarily independent from the MOE odorant receptor genes (Buck and Axel, 1991) and from the family of putative pheromone receptor genes that was previously identified in $G\alpha_{i2}^+$ VNO neurons ($G\alpha_{i2}$-VN receptors) (Dulac and Axel, 1995). G_o-VN receptor transcripts are found exclusively in the vomeronasal neuroepithelium and are not detected in the brain, the MOE, and in nonneuronal tissues. We have shown that different members of the G_o-VN gene family are expressed by small and nonoverlapping subsets of G_o^+ vomeronasal neurons and that individual sensory neurons are likely to express only one receptor gene. The corresponding receptor gene family has simultaneously been identified in mouse (see accompanying paper, Matsunami and Buck, 1997 [this issue of *Cell*]).

Thus, the G_o-VN gene family meets the set of criteria expected for mammalian pheromone receptor genes. Functional experiments will be required to demonstrate directly whether G_o-VN and $G\alpha_{i2}$-VN receptors are indeed

able to interact with chemical cues involved in pheromone response.

Expression of the G_o-VN Family of Receptors in Different Animal Species

A large range of vertebrate species communicates with pheromones in order to coordinate behaviors and reproduction. The possibility of pheromone perception in humans is still debated: the consequences of VNO activation in humans have been difficult to demonstrate, since pheromones are likely to elicit subtle changes in brain activity that are tempered by strong behavioral constraints (Humphrey, 1940; but also see Berliner et al., 1996). We had previously characterized two members of the $G\alpha_{i2}$-VN family of receptors in the human genome that appeared to be pseudogenes (Dulac and Axel, 1995). In preliminary experiments, we have now isolated ten genes sharing sequence homologies with the G_o-VN family of pheromone receptors. However, the presence of introns in the genome makes it difficult at present to identify the entire coding sequence and assess the functionality of these genes.

The Vomeronasal Organ Retains Two Independent Collections of Sensory Neurons

We have shown that G_o^+ VNO neurons are likely to express only one receptor gene of the G_o-VN family and that expression of this family of receptor is restricted to the subset of VNO neurons coexpressing G_o and is not detected in $G\alpha_{i2}$-expressing cells. Thus, distinct pheromone signals are likely to elicit electrical stimulation of restricted populations of VNO sensory neurons in order to generate distinct behavioral responses. Although there is no sharp boundary between the two neuronal populations (see Figures 2A and 2C), differential expression of several markers, including lectins (Ichikawa et al., 1994), carbohydrate epitopes (Imamura et al., 1985; Mori, 1987), and G proteins G_o and $G\alpha_{i2}$ (Halpern et al., 1995; Berghard and Buck, 1996), discriminates the apical from the basal half of the VNO neuroepithelium and distinguishes axonal fibers as they reach the anterior versus the posterior half of the accessory bulb. Cosegregation of G_o and $G\alpha_{i2}$ with two unrelated families of pheromone receptors is particularly informative because it suggests the existence of two divergent signal transduction pathways employed by spatially segregated neuronal networks. These data support the concept that the VNO evolved two separate functional units in order to detect and process pheromone signals.

The G_o-VN Family of Putative Pheromone Receptors

Protein sequences indicate that the G_o-VN receptor family shares similarities with a large group of G protein–coupled receptors that includes the Ca^{2+}-sensing receptor and metabotropic glutamate (mGluR) and GABAb receptors (Houamed et al., 1991; Masu et al., 1991; Brown et al., 1993; Pollak et al., 1993; Kaupmann et al., 1997). Thus, it seems most likely that the G_o-VN putative pheromone receptors adopt a similar molecular architecture with a large extracellular N-terminal region involved in ligand recognition preceding seven closely spaced transmembrane domains. High variability in the N-terminal domain and adjacent first transmembrane domain are consistent with the predicted ability of the G_o-VN family to recognize various classes of ligands.

Structural characteristics of G_o-VN receptors contrast vividly with those of the olfactory and pheromone receptors identified so far in vertebrates and Caenorhabditis elegans (Buck and Axel, 1991; Dulac and Axel, 1995; Troemel et al., 1995; Sengupta et al., 1996). These large families of genes encode potential surface receptors with seven highly variable transmembrane domains surrounded by a short N-terminal segment and interconnecting loops. On the basis of structural homologies with β-adrenergic receptor, it is thought that in this type of receptor, receptor–ligand interaction occurs within the plane of the membrane (Strader et al., 1987; Kobilka et al., 1988).

The Relationship between the Two Populations of VNO Sensory Neurons

Does the existence of structurally distinct pheromone receptors reflect structural necessity to accommodate divergent classes of ligands? Many chemical cues involved in pheromone response have not yet been isolated, and mammalian pheromones identified so far show extremely diverse chemical structures that include hydrophobic molecules such as steroid metabolites, prostaglandins, and fatty acid chains together with peptides and proteins (Melrose et al., 1971; Henzel et al., 1988; Sorensen et al., 1988; Jiang et al., 1990; Menzies et al., 1992; Kikuyama et al., 1995; Rasmussen et al., 1996). Moreover, receptors displaying the same molecular architecture as G_o-VN and $G\alpha_{i2}$-VN receptors (Strader et al., 1994) interact with a large variety of structural classes of ligands. Therefore, no assumption can be made concerning the type of pheromone that each class of pheromone receptors would preferentially recognize.

Alternatively, the presence of two evolutionary unrelated families of pheromone receptors might reflect the existence of independent, although not necessarily structurally different, pheromonal cues involved in distinct physiological processes. According to this model, the two sets of VNO neurons connected to either the anterior or the posterior half of the accessory bulb would ultimately project to a distinct area of the limbic system involved in different behavioral or endocrine responses. What could be the physiological basis for a dual structure of the VNO? A classical dichotomy in pheromone-mediated signals has distinguished "releasing" pheromones, inducing immediate behavioral changes such as mating or aggressive behaviors, from "priming" pheromones, which elicit long-term and mostly endocrine modifications like induction or inhibition of female estrous (Halpern, 1987). This concept has been primarily defined in insects (Wilson, 1963), and although most pheromone-induced behaviors in mammals are closely associated with endocrine changes (reviewed by Wysocki, 1989), it is conceivable that releasing and priming responses are mediated by distinct neuronal networks linked to segregated populations of VNO sensory neurons. The dual structure of the VNO might also be involved in the segregation of signals driving reproductive

and nonreproductive or parenting behaviors, species, and individual recognition.

It is also possible that two evolutionarily independent sets of sensory neurons have been selected in order to cooperate in a given physiological response. Examples of complex behaviors involving multiple chemical cues and/or multiple regions of the brain have been well documented (Murphy and Schneider, 1970; Scheller et al., 1982; Singer et al., 1986).

The Molecular Topography of the Vomeronasal System

The existence of two segregated populations of VNO neurons that express distinct families of pheromone receptor genes $G\alpha_{i2}$-VN and G_o-VN and that project to a distinct area of the accessory bulb demonstrates a first level of topographical organization in the vomeronasal system. In addition, we have found that expression of the G_o-VN family of receptor genes is regionalized into two to three distinct hemiconcentric zones of different radii, such that in each of these zones, $G_o{}^+$ neurons are able to express only a subset of the entire repertoire of G_o-VN receptor genes. Olfactory neurons are organized into four zones of receptor expression in the main olfactory epithelium, such that each zone of the epithelium is likely to project to one of four quadrants of the main olfactory bulb (Ressler et al., 1993, 1994; Vassar et al., 1993, 1994). In a similar manner, the mammalian vomeronasal system might be compartmentalized into anatomically and functionally discrete units of lesser complexity, each involved in different behavioral or endocrine responses.

Molecular Basis for Sexual Dimorphism and Age-Related Differences in Pheromone Perception

The repertoires of innate and stereotyped behaviors and hormonal changes induced by pheromones are strikingly different in males, females, and sexually immature animals (reviewed by Halpern, 1987; Wysocki, 1989). The sexual dimorphism and age-related differences in pheromone perception might result from the different processings by the brain of a same VNO stimulation and/or from variations in the VNO sensitivity itself. We have demonstrated that subsets of genes encoding the G_o-VN putative pheromone receptors present remarkable differences in their distribution in VNOs dissected from males, females, and young animals. This provides a molecular basis for differential activity in the VNO depending on the gender and the degree of sexual maturity of the animal. In addition, the difference observed in G_o-VN2 distribution in adult males or females reinforces the conceptual importance of the organization of the VNO into discrete functional units.

Finally, the regionalization of G_o-VN receptor distribution suggests the existence of positional information governing specific receptor expression. The acquisition of a precise pattern of receptor distribution during sexual maturation reveals a mechanism of refinement in the organization of the vomeronasal neuroepithelium during puberty that is consistent with the acquisition in adults of new characteristics in pheromone perception leading to behavior.

Experimental Procedures

Preparation of cDNA Libraries from Isolated VNO Neurons

VNOs were dissected from adult (7- to 8-week-old) male Lewis rats (Sprague-Dawley). Single-cell cDNA synthesis and amplification were performed and checked according to Dulac and Axel (1995). Southern blot analysis of single-cell cDNA was used to detect expression of tubulin, OMP, G_o, and $G\alpha_{i2}$ (Dulac and Axel, 1995). Eighteen cDNAs showed strong hybridization with tubulin and OMP probes, indicating that they originated from mature neurons, and were selected for further study.

Cells VN3 and VN13 exhibited high levels of G_o expression, whereas VN10 showed presence of $G\alpha_{i2}$, indicating the origin of these cells from two distinct regions of the VNO neuroepithelium. VN13 single-cell cDNA library was prepared according to Dulac and Axel.

Differential Screening of Single-Cell Library

Plaque-forming units (12×10^3) from the VN13 library were plated at low density, and duplicate filters (Hybond N^+, Amersham) were hybridized with probes generated from VN10 and VN13 single-cell cDNAs, following the procedure described in Dulac and Axel (1995). Ten phage plaques were detected that showed a positive signal unique to the VN13 probe. These plaques were purified, and the corresponding phage inserts were amplified by PCR, run on 1.5% agarose gel, blotted onto nylon filter, and hybridized with the VN10, VN3, and VN13 single-cell cDNA probes.

Isolation and Analysis of Full-Length cDNA Clones

A 425 bp clone, G_o-VN13A, present at the frequency of 0.1% in the VN13 single-cell cDNA library, was selected and in vivo excised to generate the pBlueScriptSK(−) phagemid. High stringency (65°C) screening of a cDNA library prepared from female rat VNO (Dulac and Axel, 1995) with the G_o-VN13A cDNA probe led to the isolation of G_o-VN13B, presenting 90% sequence homology with G_o-VN13A. Phages (7.2×10^5) of the female rat VNO library were further screened with the G_o-VN13B cDNA probe under low stringency conditions: hybridization was carried out at 55°C for 24 hr, and the filters were washed three times at 55°C for 30 min in 0.5× SSC and 0.5% SDS. A total of 75 positive phages were identified and the corresponding inserts were amplified by PCR and analyzed by Southern blot using the G_o-VN13B probe at both high (65°C) and low (55°C) stringency. This led to the identification of 22 cDNA clones with insert sizes longer than 3 kb. Among those, six distinct subfamilies were defined by absence of cross-hybridization under stringent conditions of hybridization and washing. Full-length clones (G_o-VN1 to G_o-VN6), each representative of a subfamily, were selected for in vivo excision and sequenced. G_o-VN13C and G_o-VN13B are identical sequences differing by a 150 bp deletion in G_o-VN13C. This sequence encodes for NMDQCANCPEYQYANTEKNKCIQKGVIVLSYE DPLGMALALIAFCFSAFTV in G_o-VN13B and is replaced by an M at position 552 in G_o-VN13C.

DNA Sequencing and Sequence Analysis

DNA sequencing was performed using ABI Prism dye terminator cycle ready reaction (Perkin Elmer) according to manufacturer's protocol. Samples were run on an ABI Prism 310 Genetic Analyzer (Perkin Elmer).

Sequence homologies were determined using the BLAST system (NIH network service). Pairwise and ClustalW alignments (BLOSUM30 matrix setting) as well as Kyte-Doolittle hydropathic analysis were obtained with the MacVector sequence analysis software (Oxford Molecular Group).

In Situ Hybridization Analysis

In situ hybridization was performed as described elsewhere (Schaeren-Wiemers and Gerfin-Moser, 1993). VNOs were dissected from adult male (8- to 9-week-old), adult female (9- to 11-week-old), and young (1-week-old) rats. Tissues were embedded in Tissue-Tek

OCT compound (Sakura). Antisens and sens digoxigenin-labeled probes were generated from the full-length cDNAs encoding for G_o, $G\alpha_{i2}$, G_o-VN13B, and G_o-VN1 to G_o-VN6, as well as from the 3′ untranslated regions of the G_o-VN1 to G_o-VN6 clones.

Imaging Processing and Statistical Analysis

Digital photographs were captured with a Leitz DMRB microscope (Leica) coupled to a ProgRes3012 digital camera (Kontron Electronic) and further processed with the Photoshop (Adobe System) and Canvas (Deneba) softwares for Macintosh. The relative positions of cells exhibiting a positive signal by in situ hybridization were measured along the basal–apical axis using the NIH Image analysis software. The number of cells in hemiconcentric sections of 10% along this axis from the basal (value = 0) to the apical (value = 100) boundaries was determined. Average data for G_o-VN1 and G_o-VN3 to G_o-VN6 were obtained from six to eight VNO sections, corresponding to four individuals analyzed in two independent experiments. For G_o-VN2, 14 VNO sections, corresponding to ten individuals and four independent experiments, were analyzed for each sex.

Southern Blot Analysis of Rat Genomic DNA and Screening of Rat and Human Genomic Libraries

Genomic DNA, prepared from Lewis rat (Sprague-Dawley) liver, was digested with the restriction enzymes EcoRI and BamHI, size fractionated on 0.8% agarose gels, and blotted onto nylon membrane (Sambrook et al., 1989). Membranes were cross-linked under UV light, hybridized overnight at both high (68°C) and low (55°C) stringency in hybridization buffer, and washed as described above. ^{32}P-labeled probes were generated by random priming, using the following DNA templates: EcoRI-EcoRV, NotI-NsiI, EcoRI-SalI, PstI-NdeI, XbaI-HincII, and EcoRI-NsiI fragments of G_o-VN1 to G_o-VN6, respectively; a full-length (425 bp) insert of G_o-VN13A; and a cDNA fragment including the seven transmembrane domains of G_o-VN13B.

Plaque-forming units (3×10^5) from rat and human genomic libraries (Stratagene) were screened at low stringency (55°C) using a mix of ^{32}P-labeled probes prepared from fragments of G_o-VN1 to G_o-VN6 encompassing the transmembrane domains 2 to 7.

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